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# IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN FOLLICLE STIMULATING HORMONE AND THE FOLLICLE STIMULATING HORMONE RECEPTOR

Johann Sohn  
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ABSTRACT OF DISSERTATION

Johann Sohn

The Graduate School  
University of Kentucky

2005

**IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES  
BETWEEN HUMAN FOLLICLE STIMULATING HORMONE AND THE  
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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the degree of Doctor of Philosophy in the  
College of Arts and Sciences  
at the University of Kentucky

By

Johann Sohn

Lexington, Kentucky

Director: Dr. Tae H. Ji, Professor of Chemistry

Lexington, Kentucky

2005

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## ABSTRACT OF DISSERTATION

### **IDENTIFICATION AND CHARACTERIZATION OF CONTACT SITES BETWEEN HUMAN FOLLICLE STIMULATING HORMONE AND THE FOLLICLE STIMULATING HORMONE RECEPTOR**

Follicle stimulating hormone (FSH) comprises an  $\alpha$  subunit and a  $\beta$  subunit, whereas the FSH receptor consists of two halves with distinct functions, the N-terminal extracellular exodomain and C-terminal membrane associated endodomain. FSH initially binds to exodomain, and the resulting FSH/exodomain complex modulates the endodomain and generates signal. However, it has been difficult to determine which subunit of FSH contacts the exodomain or endodomain, and in what orientation FSH interacts with them. To address these crucial issues, the receptor was Ala-scanned and the hormone subunits were probed with photoaffinity labeling with receptor peptides corresponding to the N-terminal region of the exodomain and exoloop 3 of the endodomain. The results show that both regions of the receptors are important for hormone binding and signal generation. In addition, the FSH  $\beta$  subunit is specifically labeled with the N-terminal peptide, whereas the  $\alpha$  subunit is labeled with the exoloop 3 peptide. These contrasting results show that the FSH  $\beta$  subunit is close to the N-terminal region and the  $\alpha$  subunit is projected toward exoloop 3 in the endodomain. The results raise the fundamental question whether the  $\alpha$  subunit, common among the glycoprotein hormones, plays a major role in generating the hormone signal common to all glycoprotein hormones.

**KEYWORDS:** FSH, FSH Receptor, GPCR, photoaffinity labeling

Johann Sohn  
April 18, 2005

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BETWEEN HUMAN FOLLICLE STIMULATING HORMONE AND THE  
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By

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Director of Graduate Studies

April 18, 2005

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DISSERTATION

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The Graduate School

University of Kentucky

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“The LORD is my shepherd, I shall not be in want. He makes me lie down in green pastures, he leads me beside quiet waters, he restores my soul. He guides me in paths of righteousness for his name’s sake. Even though I walk through the valley of the shadow of death, I will fear no evil, for you are with me; your rod and your staff, they comfort me. You prepare a table before me in the presence of my enemies. You anoint my head with oil; my cup overflows. Surely goodness and love will follow me all the days of my life, and I will dwell in the house of the LORD forever.” (Psalms 23)

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## **Chapter 1 Introduction**

### **Gonadotropins**

Follicle-stimulating hormone (FSH), luteinizing hormone (LH), and chorionic gonadotropin (CG) are gonadotropins (Bousfield et al., 1996). These hormones belong to the family of glycoprotein hormones which also includes thyroid-stimulating hormone (TSH) (Pierce and Parson, 1981). The gonadotropins are produced by different tissues: FSH and LH of pituitary origin; CG of placental origin (Pierce and Parson, 1981).

FSH has been found in animals as a key hormonal molecule playing a central role in the regulation of mammalian reproduction. In females, it is essential to promote and sustain ovarian and follicular development and maturation since early development stages, whereas in males it regulates spermatogenesis (Adashi, 1996; Hsueh et al., 1983). FSH binds specifically to follicle-stimulating hormone receptors (FSHR) on granulosa cells in the ovary or Sertoli cells in the testis and stimulates the aromatase system that catalyzes the conversion of androgens into estrogens (Adashi, 1996).

LH is also a key molecule for reproduction. The function of LH is the initiation of steroidogenesis in the thecal cells of the follicle and the Leydig cells of the testis. This glycoprotein hormone also induces the ovulation and luteinization of fully developed follicles. LH and FSH have synergistic effects on estrogen synthesis in the ovary, due to the fact that FSH promotes the production of estrogens from androgens. LH is involved in maintaining corpus luteum progesterone production, although this role appears to vary among species. In males, LH triggers the synthesis of androgens needed for spermatogenesis and the development of secondary sexual characteristics. (Catt et al., 1980; Filicori, 1999; Richards et al., 1995).

It is evident that physiologic growth and maturation in reproduction is dependent on the dynamic interplay between both gonadotropins; key physiologic events rely on both the prevalence of either FSH or LH activity.

CG is closely related in structure and activity to LH, since CG and LH bind to the same receptor, LH/CG receptor. CG plays an important role for maintaining pregnancy in animals. Secretion of human chorionic gonadotropin (hCG) from placenta begins at about the time of implantation and reaches peak levels during the first trimester (Cameo et al., 2004; Keay et al., 2004). hCG serves as a signal to the ovary to maintain the corpus luteum thus prolonging luteal progesterone synthesis. hCG binds to LH/CG receptors expressed on the surface of luteal cells and granulosa cells, resulting in the activation of adenylyl cyclase and production of intracellular cAMP (Menon and Kiburz, 1974). In the absence of hCG, the decline in maternal LH due to inhibition of the pituitary by progesterone would result in menstruation and spontaneous abortion of the embryo (Keay et al., 2004).

Structurally, each of the gonadotropins is comprised of two subunits, a common alpha subunit and a hormone-specific beta subunit. The alpha subunit consists of 92 amino acids and the composition of the beta subunit varies from hormone to hormone (FSH: 111 a.a., LH: 121 a.a., CG: 145 a.a., respectively). The beta subunits share considerable structural homology and are thought to have evolved from a common precursor (Pierce and Parson, 1981).

The 9.4 kb human  $\alpha$  subunit gene on chromosome 6q is composed of four exons, which encodes a 92 amino acid protein (Fiddes and Goodman, 1981), and the 10 kb human FSH  $\beta$  gene localized to chromosome 11p, contains three exons and encodes a 111 amino acid subunit (Jameson et al., 1988) (Figure 1.1). Each subunit contains two glycosylation

sites: at Asn52 and Asn78 in the  $\alpha$  subunit and at conserved sites in the  $\beta$  subunit, Asn7 and Asn24 in human FSH (hFSH) (Gharib et al., 1990). Only the  $\alpha\beta$  dimer is capable of high affinity binding to receptors and inducing biological effects. The dissociated subunits lose high affinity binding activity (Roche and Ryan, 1985). Both of the  $\alpha\beta$  subunits of FSH interact with its receptor.

### **The Follicle-Stimulating hormone (FSH) Receptor**

The follicle-stimulating hormone receptor (FSHR) belongs to a subfamily of glycoprotein hormone receptors within the G-protein coupled receptor family (Ji et al., 1998). FSHR is composed of two halves of ~350 amino acids, the extracellular N-terminal exodomain and membrane anchored C-terminal endodomain that includes 7 transmembrane helices and cytotail (Loosfelt et al., 1989; McFarland et al., 1989; Sprengel et al., 1990) (Figure 1.2). Glycoprotein hormones initially bind to the exodomain, and then the resulting hormone/exodomain complex modulates the endodomain (Ji et al., 1995b), which activates adenylyl cyclase (AC) to generate cAMP and phospholipase C $\beta$  (PLC $\beta$ ) to produce inositol phosphate and diacylglycerol (Figure 1.3). Thus, the orchestrated interactions among the hormone, exodomain and endodomain are critical for successful signal transduction. Little mechanisms on the subject, particularly concerning the FSH receptor, have been demonstrated to explain the receptor activation.

The exodomain is important for binding the hormone with high affinity (Ji and Ji, 1991; Tsai-Morris et al., 1990; Xie et al., 1990) without hormone action (Ji and Ji, 1991; Remy et al., 1993). The exodomain and hormone complex undergoes a conformational

change and is thought to modulate the endodomain (Ji et al., 1995a), thus generating a signal. The high affinity interaction of the exodomain and FSH is the essential first step guiding to signal transduction and hormone action. Regardless of the importance of this initial binding event, only restricted information is available concerning the particular contact sites in the exodomain as well as the hormone.

The exodomain consists of 8 - 9 Leu rich repeats (Bhowmick et al., 1996; Koo et al., 1991; McFarland et al., 1989), which make up the bulk of the exodomain and are flanked by a short N-terminal Cysteine cluster and a C-terminal hinge region (Zeng et al., 2001). These Leu rich repeats are commonly thought to construct a concave structure (Jiang et al., 1995) (Figure 1.2).

Although the Leu rich repeats interact with the hormone (Song et al., 2001a), it has been suggested that the short N-terminal region flanking the primary hormone binding Leu rich repeats is crucial for FSH binding (Dias et al., 1998). This region contains sites which appear critical for not only hormone binding but receptor trafficking as well (Nechamen and Dias, 2000). Moreover, it has been shown that accessibility of the N-terminal residues 15-44 is decreased upon binding (Schmidt et al., 2001). Such an important hormone contact site outside of the Leu rich repeats could provide new insights of FSH-receptor interaction and signal generation. In fact, a similar site has been identified in the N-terminal region of LHR (Hong et al., 1998; Phang et al., 1998). Although there is little sequence homology in the N-terminal regions of FSHR and LHR, the conservation of a Cysteine cluster suggests a comparable role in receptor binding, albeit with differential specificity.

In addition, it has been reported that FSH and hCG binding to their cognate receptors is modulated by certain amino acid residues of exoloops 2 and 3 of the endodomain (Ryu et al., 1998a; Ryu et al., 1998b). Moreover, the hinge region of the exodomain has been shown to interact with exoloop 2 of the endodomain and modulates cAMP induction (Nakabayashi

et al., 2000; Nishi et al., 2002; Zeng et al., 2001). These results suggest that there is the interaction and modulation between the exodomain and the exoloops of the endodomain and it is required for signal generation. Nevertheless, there is no experimental evidence whether the hormone complexed with the exodomain also contacts the exoloops.

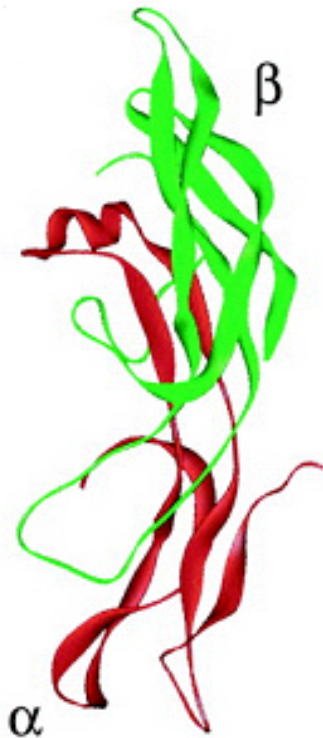
## **Goal of Dissertation**

Sixteen percent of the human population are infertile, affecting 80 million people worldwide with 5 million in the United States alone (Forti and Krausz, 1998; Page, 1989). The receptor and hormone could potentially be used as fertility drugs as well as contraceptives. Current contraceptives are steroid and have serious side effects since steroids can affect most of body cells. In contrast, gonadotropin receptors are found primarily in the reproductive organs. Therefore, the drugs based on gonadotropins will have a higher specificity and less side effects. The hormone-receptor interactions generate signals and induce the hormone action, so it is essential to identify the contact points between hormone and receptor. Further understanding of the hormone and receptor interactions will provide new insights in targeting drug design and understanding the basic mechanism of GPCR activation.

The goal of this dissertation work is to further describe the contact sites between FSH and the FSH receptor. FSH initially binds to exodomain, and the resulting FSH/exodomain complex modulates the endodomain and generates signal. However, it has been difficult to determine which subunit of FSH contacts the exodomain or endodomain and in what orientation FSH interacts with them. Photoaffinity labeling studies examine the contribution

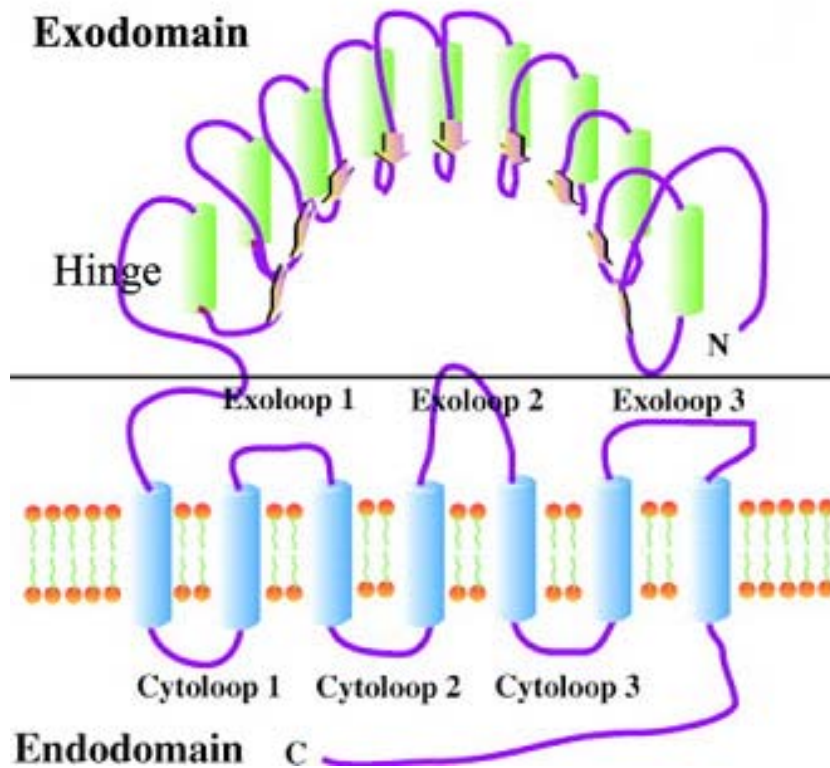


of N-terminal and exoloop 3 of receptor to hormone binding. These results provide new insights into the mechanism of hormone/exodomain/endodomain interaction.



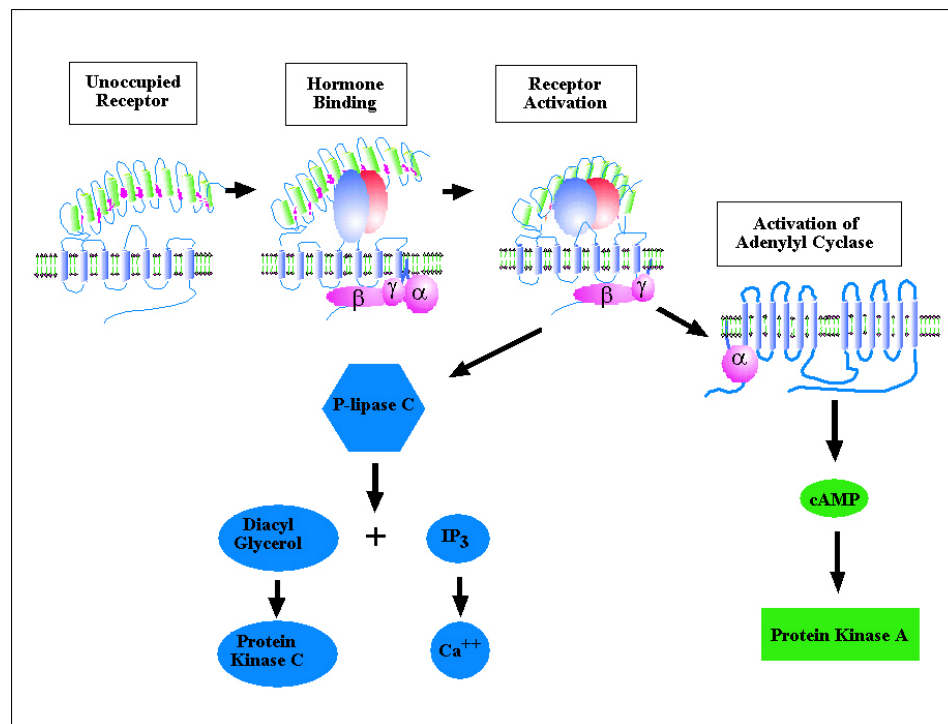
**Figure 1.1 Crystal structure of FSH.**

FSH is a heterodimer composed by two different subunits:  $\alpha$  and  $\beta$ . The FSH  $\alpha$  subunit (92 amino acids) is common to all gonadotropins (shown in red), and the FSH  $\beta$  subunit (111 amino acids) is specific for FSH (shown in green).



**Figure 1.2 Structure of the FSH receptor.**

A schematic drawing of FSHR with two distinct domains, exodomain and endodomain. The N-terminal exodomain consists of ~350 amino acids and binds to hormone with high affinity. An equal-sized endodomain is membrane-associated and is responsible for signal generation.



**Figure 1.3 Model of signal transduction pathways of the FSH receptor.**

FSH initially binds to the exodomain, and then the resulting hormone/exodomain complex modulates the endodomain, which activates adenylyl cyclase (AC) to generate cAMP and phospholipase C $\beta$  (PLC $\beta$ ) to produce inositol phosphate and diacylglycerol.

## **Chapter 2 Interaction of Follicle-stimulating Hormone with Exoloop 3 of the FSHR**

### **INTRODUCTION**

The follicle-stimulating hormone receptor (FSHR) and other glycoprotein hormone (LH/CG and TSH) receptors belong to a structurally unique subfamily of G protein-coupled receptors. Unlike other receptor subfamilies, they comprise two equal halves, an N-terminal extracellular half (exodomain) and a C-terminal membrane associated half (endodomain) (Loosfelt et al., 1989; McFarland et al., 1989; Nagayama et al., 1989; Sprengel et al., 1990). The exodomain is ~350 amino acids long and alone is capable of high affinity hormone binding (Davis et al., 1995; Ji and Ji, 1991; Tsai-Morris et al., 1990; Xie et al., 1990) with hormone selectivity (Braun et al., 1991; Liu et al., 1994; Moyle et al., 1994) but without hormone action (Ji and Ji, 1991) (Remy et al., 1993). Receptor activation occurs in the endodomain (Ji et al., 1995b) which is structurally equivalent to the entire molecule of many other G protein-coupled receptors (Probst et al., 1992). Glycoprotein hormones initially bind to the exodomain, and then the resulting hormone/exodomain complex modulates the endodomain (Ji et al., 1995b), which activates adenylyl cyclase (AC) to generate cAMP and phospholipase C $\beta$  (PLC $\beta$ ) to produce inositol phosphate and diacylglycerol. Therefore, the ternary interactions among the hormone, exodomain and endodomain are crucial for successful signal generation. However, there is little information on the subject, particularly concerning the FSH receptor. Since the exodomain lacking the endodomain is capable of high affinity hormone binding (Davis et al., 1995; Ji and Ji, 1991; Ryu et al., 1998a; Tsai-Morris et al., 1990; Xie et al., 1990), the high affinity hormone binding appears to be independent of the endodomain. Contrary to this view, it has been reported that FSH and hCG binding to their cognate receptors is regulated by certain residues of exoloops 2 and 3 of the endodomain (Ryu et al., 1998a; Ryu et al., 1998b).

Furthermore, the hinge region of the exodomain interacts with exoloop 2 and modulates cAMP induction (Nakabayashi et al., 2000; Nishi et al., 2002; Zeng et al., 2001). These results suggest that the exodomain interacts with the exoloops and modulates them for signal generation. Yet, it is unclear whether the hormone complexed with the exodomain also contacts the exoloops.

In this study, I set out to investigate whether exoloops interact with the hormone at all. In a step toward this goal, I examined exoloop 3 of FSHR for its involvement in the interaction with FSH. It is the shortest of the 3 exoloops, consisting of 11 amino acids (Figure 2.1), and has been implicated in the cAMP signal generation (Angelova et al., 2002; Ryu et al., 1998a; Ryu et al., 1996). These observations show, for the first time, the interaction of exoloop 3 with FSH, in particular the FSH  $\alpha$  subunit, the mode of this interactions (Sohn et al., 2002).

## **EXPERIMENTAL PROCEDURES**

### **Materials**

Human FSH was purchased from the National Hormone and Pituitary Program. Denatured FSH was prepared by boiling the hormone in 8 M urea for 30 min.

### **Derivatization and radioiodination of peptide**

A peptide mimic corresponding to the exoloop 3 sequence of K<sup>580</sup>VPLITVSKAK<sup>590</sup> (FSHR<sup>exo3</sup>) was synthesized, to which a Tyr residue was attached to the C-terminus for radioiodination. The N-terminus of the peptide was acetylated and the C-terminus amidated. NHS-ABG was synthesized as previously described (Ji and Ji, 1981) and freshly dissolved in dimethyl sulfoxide to a concentration of 50 mM and NHS-ABG in 0.1 M sodium

phosphate (pH 7.5) to a concentration of 20 mM. These reagent solutions were immediately used to derivatize receptor peptides. In the dark, 10  $\mu$ l of NHS-ABG was added to 30  $\mu$ g of receptor peptides in 40  $\mu$ l of 0.1 M sodium phosphate (pH 7.5). The mixture was incubated with NHS-ABG for 30 min at 25° C. The following were added to the derivatization mixture: 1 mCi of Na<sup>125</sup>I-iodine in 10  $\mu$ l of 0.1 M NaOH and 7  $\mu$ l of chloramine-T (1 mg/ml) in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4. After 20 sec, 7  $\mu$ l of sodium metabisulfite (2.5 mg/ml) in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, was introduced to terminate radioiodination. Derivatized and radioiodinated ABG-<sup>125</sup>I-FSHR<sup>exo3</sup> solution was mixed with 60  $\mu$ l of 16% sucrose solution in PBS and fractionated on Sephadex Superfine G-10 column (0.6 X 15 cm) using PBS.

### **Photoaffinity labeling of FSH**

The following solutions were sequentially introduced to siliconized glass tubes: 20  $\mu$ l of 0.9% NaCl and 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4 (PBS), 10  $\mu$ l of FSH (10 ng/ $\mu$ l) in PBS, and 10  $\mu$ l of ABG-<sup>125</sup>I-FSHR<sup>9-38</sup> or <sup>125</sup>I-FSHR<sup>Bpa13</sup> (10 ng/ $\mu$ l) in PBS. Competitive inhibition experiments were carried out as described for the photoaffinity labeling experiments, except that 10  $\mu$ l instead of 20  $\mu$ l of PBS was introduced to each tube and the mixture was incubated with 10  $\mu$ l of increasing concentrations of nonradioactive receptor peptides. The mixtures were incubated at 37° C for 90 min in the dark, irradiated with Mineralight R-52 UV lamp for 3 minutes as previously described (Ji and Ji, 1980), and solubilized in 2% SDS, 100 mM dithiothreitol and 8 M urea. The samples were electrophoresed on 8-12% polyacrylamide gradient gels. Gels were dried on filter paper and exposed to an imaging screen overnight, which was scanned on a phosphorimager.

### **<sup>125</sup>I-FSH Binding assay**

wtFSHR cDNAs were prepared in the pSELECT vector, sequenced on a Beckman CEQ 2000XL capillary sequencer, subcloned into pcDNA3 (Invitrogen) as described previously (Ji and Ji, 1993). Wild type receptor constructs were transfected into HEK 293 cells by the calcium phosphate method as described previously (Ji and Ji, 1993). Stable cell lines were established in minimum essential medium containing 10% horse serum and 500 µg/ml G418. These cells were used for hormone-binding. All of the assays were carried out in duplicate and repeated 4–5 times, and the means ± S.D. were calculated. wtFSHR-expressing stable cells were assayed for <sup>125</sup>I-FSH binding in the presence of 100,000 cpm of <sup>125</sup>I-FSH (Ji and Ji, 1981) and increasing concentrations of unlabeled FSH or exoloop 3 peptide.

### **Deglycosylation**

The FSH α and β subunits co-migrate on SDS-PAGE. To separate them on the gel FSH was deglycosylated with PNGase F before and after it was photoaffinity labeled. Enzymatic cleavage was done by incubation of the labeled FSH complex with 20 or 50 units of PNGase F (New England BioLabs, Inc., MA) in 40 µl for 18 h at 37° C. The samples were solubilized in SDS under the reducing condition and electrophoresed on 15 % gel containing 9 M urea.



## RESULTS

### Photoaffinity labeling of FSH with exoloop 3 peptide

To test the possible interaction of exoloop 3 with the hormone,  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> was incubated with FSH and irradiated with UV for increasing time periods. Samples were solubilized in SDS under the reducing condition and electrophoresed. The autoradiographic phosphorimage of the gel (Figure 2.2.A) revealed the labeling of the FSH band. The two subunits of the human FSH preparation comigrate on SDS-PAGE. The band was not labeled when the sample was not irradiated with UV, suggesting the requirement for UV irradiation. The extent of the labeling was dependent on the irradiation time, reaching the maximum after 60 s of irradiation. The results show that the labeling is saturable.

To determine the nature of the labeling, increasing concentrations of the hormone were labeled with a constant amount of  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> (Figure 2.2.B). Conversely, increasing concentrations of  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> were used to label a constant amount of FSH (Figure 2.2.C). The labeling plateaued under both conditions, indicating saturable labeling. To examine the relationship of the labeling with other exoloops and receptor peptide, FSH was incubated with  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> in the presence of increasing concentrations of unlabeled FSHR peptides corresponding to exoloops 1, 2, and 3 as well as the N-terminal sequence Ser<sup>9</sup>-Lys<sup>40</sup>, FSHR<sup>9-40</sup>, which is known to interact with FSH. Increasing concentrations of the peptides inhibited the photoaffinity labeling in a dose-dependent manner and eventually blocked the labeling with varying affinity (Figure 2.3), suggesting a specificity. FSHR<sup>exo2</sup> is the most potent inhibitor, suggesting the possibility of its strong interaction with the hormone. Furthermore,  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> failed to label denatured FSH that does not bind to the receptor, despite high concentrations of the peptide (Figure 2.4.A), suggesting the

specificity of the affinity labeling for biologically active FSH. FSH was denatured by boiling in 8 M urea for 30 min. To test whether the denatured FSH remained in solution, the mixture of radioactively labeled FSH and unlabeled FSH was denatured, varying volumes of the mixture were transferred to other tubes, and the radioactivity was counted. The transfer was quantitative with a 99-100 % efficiency, indicating that denatured FSH was present in the photoaffinity labeling tube.  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> did not label urokinase, nor did it label phospholipases A, C, and D (Figure 2.4.B). In addition, it failed to noticeably label human growth hormone (Figure 2.4.B). The exoloop 3 peptide inhibited  $^{125}\text{I}$ -FSH binding to the receptor on intact cells in a dose-dependent manner. These results show that the binding to and labeling of FSH by the peptide were specific to bioactive FSH.

Since the  $\alpha$  and  $\beta$  subunits of human FSH comigrate on SDS-PAGE, it is unclear which of the subunits was labeled. To determine the identity of the labeled subunit(s), FSH was labeled with  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup>, deglycosylated with PNGase F, and electrophoresed. The labeled band corresponded to the  $\alpha$  subunit (Figure 2.4.B). Deglycosylated human FSH separates into two bands on SDS-PAGE, the higher molecular weight  $\beta$  subunit in the upper band and the smaller  $\alpha$  subunit in the lower band, which was verified by monoclonal anti subunit antibodies (Sohn et al., 2003) as shown in Figure 3.9.

## DISCUSSION

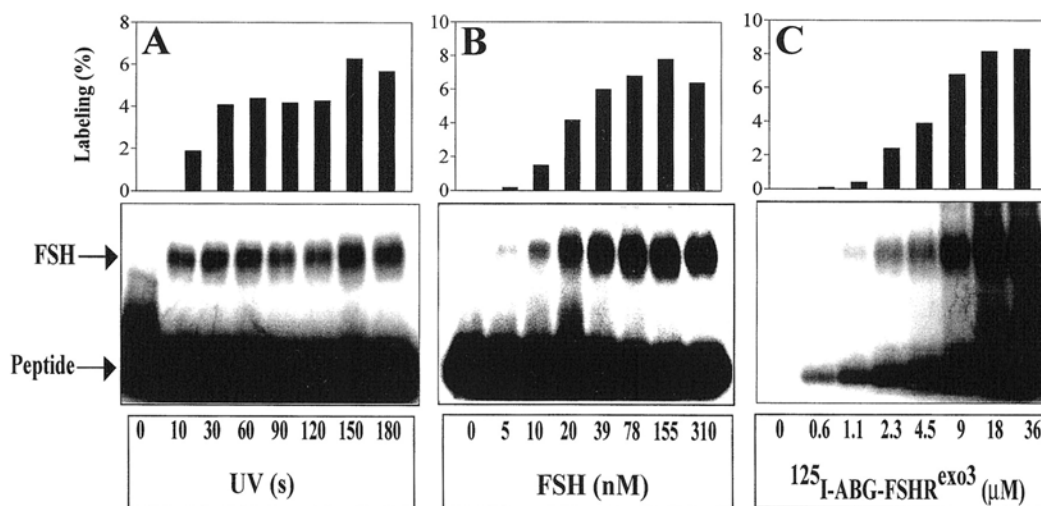
In conclusion, our observations in this study show the interaction of the FSHR exoloop 3 with FSH, specifically the  $\alpha$  subunit. This is consistent with the decade long view that the

common  $\alpha$  subunit of the glycoprotein hormones is likely to induce the common hormone action, including cAMP induction (Pierce and Parson, 1981).

FSHR (human)	KVPLITVSKAK
FSHR (Rat)	-----
FSHR (mouse)	-----
FSHR (bovine)	-----S-
FSHR (pig)	-----S-
FSHR (sheep)	-----S-
FSHR (horse)	-----S-
FSHR (chick)	R-----S-
FSHR (donkey)	--A-----S-
LHR (human)	-----TNS-
LHR (Rat)	-----TNS-
LHR (mouse)	-----TNS-
LHR (bovine)	-----TNS-
LHR (pig)	-----TNS-
LHR (sheep)	-----TNS-
LHR (carja)	-M-----TNS-
TSHR (human)	NK-----NS-
TSHR (Rat)	NK-----TNSG
TSHR (mouse)	NK-----TNS-
TSHR (bovine)	NK-----TNS-
TSHR (pig)	NK-----TNS-
TSHR (sheep)	NK-----TNS-
TSHR (Canfa)	NK-----TNS-

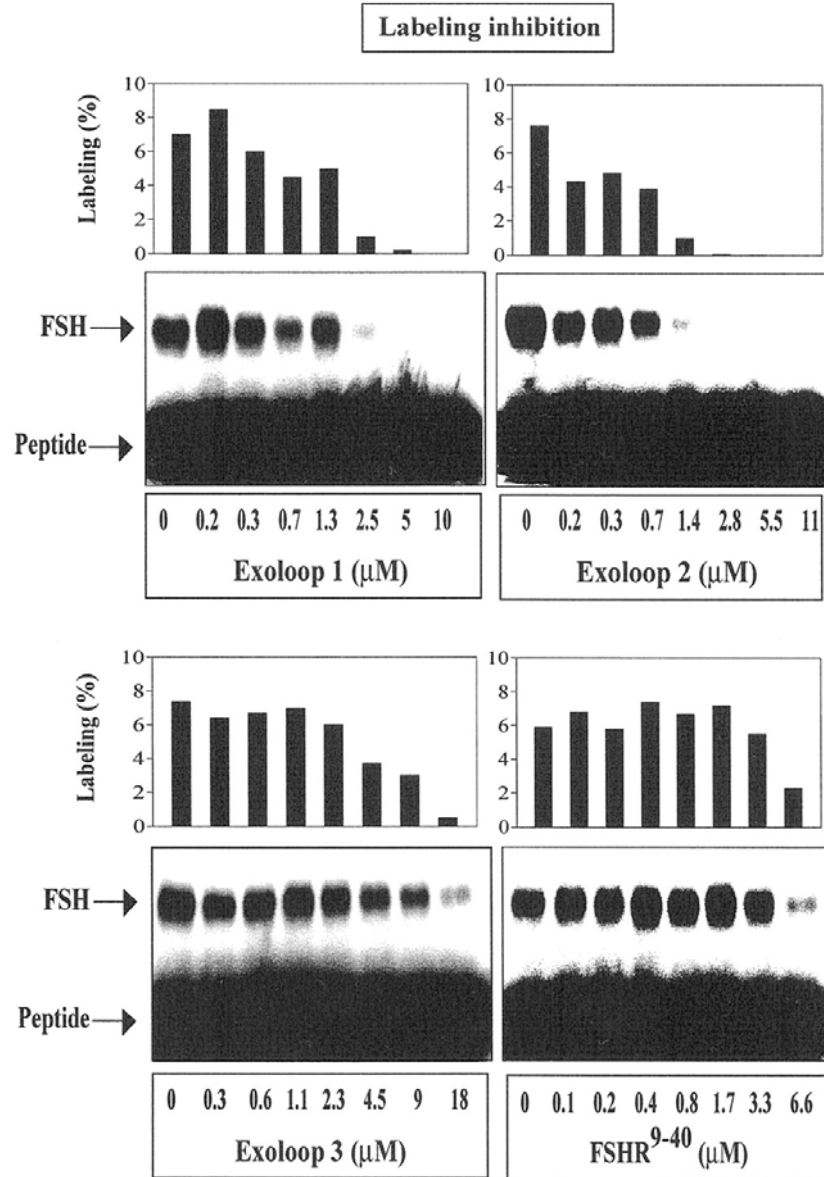
**Figure 2.1 Sequence alignment of exoloop 3.**

The exoloop 3 sequences of FSHR, luteinizing hormone receptor, and thyroid-stimulating hormone receptor were aligned among species. Identical residues are presented as dashed lines. LHR, luteinizing hormone receptor; TSHR, thyroid-stimulating hormone receptor.



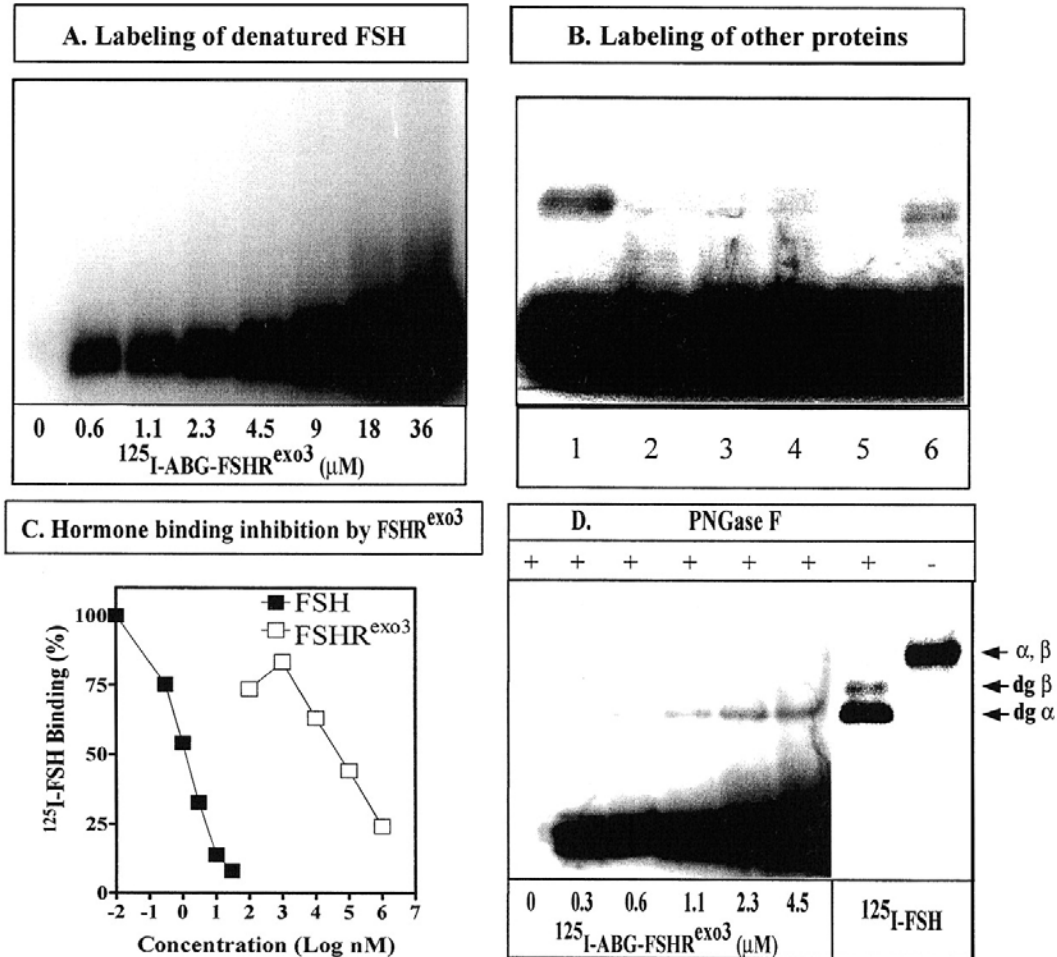
**Figure 2.2 Autoradiograph of photoaffinity-labeled FSH.**

The peptide corresponding to the FSHR exoloop 3 sequence ( $\text{FSHR}^{\text{exo3}}$ ) was synthesized, derivatized with NHS-ABG, and radioiodinated to produce  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$ . As shown in **A**, FSH was incubated with  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$  and irradiated with UV for increasing periods of time. The samples were solubilized in SDS under the reducing conditions and electrophoresed as described under "Experimental Procedures." After electrophoresis, the gel was dried and autoradiographed using phosphorimaging device. The intensity of each band in a gel lane was measured, and the percentage of the labeled FSH band in a gel lane was calculated based on the total intensity of a gel lane and presented in the bar graph. As shown in **B**, increasing concentrations of FSH were incubated with a constant amount of  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$  and irradiated with UV for 90 s. As shown in **C**, increasing concentrations of  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$  were incubated with a constant amount of FSH and irradiated with UV for 90 s.



**Figure 2.3 Competitive inhibition of photoaffinity labeling.**

FSH was photoaffinity-labeled with  $^{125}\text{I}$ -AB-FSHR<sup>exo3</sup>, as described in the legend for Figure 2.2, in the presence of increasing concentrations of unlabeled competitor peptides, exoloop 1 peptide (A), exoloop 2 peptide (B), exoloop 3 peptide (C), and FSHR<sup>9-40</sup> (D).



**Figure 2.4 Identification of the labeled FSH subunit and futile labeling of denatured FSH.**

As shown in panel **A**, denatured FSH that is not capable of binding and activating FSHR was labeled with increasing concentrations of  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$  as described in the legend for Figure 2.2. As shown in panel **B**,  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$  was used to photoaffinity-label FSH (lane 1), phospholipase A (lane 2), phospholipase C (lane 3), phospholipase D (lane 4), urokinase (lane 5), and human growth hormone (lane 6). As shown in panel **C**, inhibition of  $^{125}\text{I-FSH}$  binding to the receptor on intact cells in the presence of unlabeled FSH (black square) and exoloop 3 peptide (open square). As shown in panel **D**, FSH was photoaffinity-labeled with  $^{125}\text{I-AB-FSHR}^{\text{exo3}}$ , treated with PNGase F to deglycosylate it, and electrophoresed as described under "Experimental Procedures." The FSH  $\alpha$  and  $\beta$  subunits separated in the lower band and upper band, respectively.

### **Chapter 3 Orientation of Follicle-stimulating Hormone (FSH) Subunits Complexed with the FSH Receptor : Beta-Subunit Toward The N-Terminus of Exodomain And Alpha-Subunit to Exoloop 3.**

#### **INTRODUCTION**

Follicle-stimulating hormone (FSH) consists of an  $\alpha$  subunit of 92 amino acids and  $\beta$  subunit of 111 amino acids. Both of the subunits are necessary for hormone action (Jackson et al., 1999; Narayan et al., 2002). The crystal structure of human FSH (Fox et al., 2001) shows that the two subunits are tightly associated in a crescent with the C termini in the concave side and the N termini in the convex side (Figure 3.1). This is essentially the same as the human chorionic gonadotropin structure (Lapthorn et al., 1994; Wu et al., 1994). An exception to this intermingled subunit structure is the two polarized tips of the crescent: the  $\alpha$  tip consisting of the  $\alpha$  loops 1 and 3 and the  $\beta$  tip of the  $\beta$  loops 1 and 3.

In contrast to the tightly held hormone structure, the FSH receptor (FSHR), a G protein-coupled receptor, has two distinct domains as shown in Figure 3.1.B. The extracellular N-terminal exodomain comprises ~350 amino acids, and the membrane-associated C-terminal endodomain with a similar number of amino acids consists of seven transmembrane helices, three exoloops, three cytoloops, and the C-terminal cytoplasmic tail (Loosfelt et al., 1989; McFarland et al., 1989; Sprengel et al., 1990). The exodomain binds the hormone with high affinity (Ascoli et al., 2002; Babu et al., 1999; Davis et al., 1995; Ji and Ji, 1991; Schmidt et al., 2001; Seetharamaiah et al., 1994; Tsai-Morris et al., 1990; Xie et al., 1990) and selectivity (Vischer et al., 2003), whereas the hormone signal is generated in



the endodomain (Angelova et al., 2002; Ji et al., 1995b; Nishi et al., 2002; Tao et al., 2002; Zeng et al., 2001). FSH initially interacts with the exodomain, and the resulting FSH/exodomain complex modulates the endodomain to generate hormone signal. Important amino acids have been identified for the interaction of the hormone and receptor (Dias et al., 1998; Osuga et al., 1997). However, the orientation of FSH  $\alpha$  and  $\beta$  in the ternary complex of the hormone, exodomain, and endodomain has been a major enigma and difficult to determine.

The bulk of the exodomain comprises 8–9 Leu-rich repeats (LRR) (Bhowmick et al., 1996; Couture et al., 1996; Jiang et al., 1995; Koo et al., 1991; McFarland et al., 1989), which are flanked by the short upstream N-terminal region and the downstream hinge region. LRRs are thought to form a one-third of a doughnut structure (Bhowmick et al., 1996; Couture et al., 1996; Jiang et al., 1995). FSH appears to interact with LRRs (Dias et al., 1998; Song et al., 2001b) and the N-terminal and hinge regions (Nakabayashi et al., 2000; Zeng et al., 2001). However, it is unclear which subunit of FSH contacts the exodomain or endodomain and in what orientation FSH interacts with them, although the concave C-terminal side of FSH appears to interact with the receptor. For example, FSH may interact horizontally or vertically with LRRs (Figure 3.1, C and D) and in two directions. These are crucial pieces of information for understanding the interactions among the hormone, exodomain, and endodomain and the mechanistics of signal generation. In addition, the information will facilitate the design of agonists and antagonists and development of new therapeutics. Because of the importance, the interactions of glycoprotein hormones with their receptors have been modeled (Bhowmick et al., 1996; Dias et al., 1998; Fox et al., 2001; Lapthorn et al., 1994; Wu et al., 1994) based on the crystal structure of the LRRs of ribonuclease inhibitor complexed with its ligand (Kobe and Deisenhofer, 1995). However, the evidence has been elusive.

In a step to resolve this issue, I set out to distinguish the interactions of the FSH subunits with the N-terminal region of the exodomain and exoloop 3 in the endodomain. My results show the interaction of FSH  $\beta$  with the N-terminal region of the exodomain and the  $\alpha$  tip of FSH  $\alpha$  with exoloop 3 (Figure 3.1.E) (Sohn et al., 2003).

## **EXPERIMENTAL PROCEDURES**

### **Materials**

Human FSH was purchased from the National Hormone and Pituitary Program. Denatured FSH was prepared by boiling the hormone in 8 M urea for 30 min. Rabbit anti-FSH  $\alpha$  serum, rabbit anti-FSH  $\beta$  serum, and monoclonal anti-FSHR 106.105 antibody were kindly provided by Dr. James Dias. Anti-rabbit IgG conjugated with peroxidase was purchased from Pierce. Peptide mimics including wild type peptides corresponding to the Ser<sup>9</sup>-Lys<sup>40</sup> sequence (FSHR<sup>9-40</sup>) and exoloop 3 and a photoactivable peptide containing benzoyl phenylalanine (Bpa) in place of Phe<sup>13</sup> (FSHR<sup>9-40F13Bpa</sup>) were synthesized by Genemed Synthesis (San Francisco, CA) and purified on a Vydac C<sub>18</sub> high pressure liquid chromatography column using solvent gradient from 100% of 0.1% trifluoroacetic acid in water to 20% of 0.1% trifluoroacetic acid in water and 80% 1-propanol.

## **Mutagenesis and Functional Expression of FSH Receptors**

Mutant FSHR cDNAs were prepared in the pSELECT vector using the Altered Sites mutagenesis system (Promega), sequenced on a Beckman CEQ 2000XL capillary sequencer, subcloned into pcDNA3 (Invitrogen) as described previously (Ji and Ji, 1993), and sequenced again to verify mutation sequences. This procedure does not involve polymerase chain reaction and therefore does not have its infidelity problems. Wild type and mutant receptor constructs were transfected into HEK 293 cells by the calcium phosphate method as described previously (Ji and Ji, 1993). Stable cell lines were established in minimum essential medium containing 10% horse serum and 500 µg/ml G418. These cells were used for hormone-binding and cAMP production. All of the assays were carried out in duplicate and repeated 4–5 times, and the means  $\pm$  S.D. were calculated.

### **<sup>125</sup>I-FSH Binding and Intracellular cAMP Assay**

Stable cell lines were assayed for <sup>125</sup>I-FSH binding in the presence of 100,000 cpm of <sup>125</sup>I-FSH (Ji and Ji, 1981) and increasing concentrations of unlabeled FSH. The K<sub>d</sub> values were determined by Scatchard plots. For intracellular cAMP assay, cells were washed twice with Dulbecco's modified Eagle's medium and incubated in the medium containing 0.1 µg/ml isobutylmethylxanthine for 15 min. Increasing concentrations of FSH were then added, and incubation was continued for 45 min at 37 °C. After removing the medium, the cells were rinsed once with fresh medium without isobutylmethylxanthine, lysed in 70% ethanol, freeze-thawed in liquid nitrogen, and scraped. After pelleting cell debris at 16,000 x g for 10 min at 4 °C, the supernatant was collected, dried under vacuum, and resuspended in 10 µl of cAMP assay buffer (Amersham Biosciences). cAMP concentrations were determined with an <sup>125</sup>I-

cAMP assay kit (Amersham Biosciences) following the manufacturer's instructions and validated for use in our laboratory.

### **<sup>125</sup>I-FSH Binding to Solubilized FSHR**

Transfected cells were washed twice with ice-cold 150 mM NaCl, 20 mM HEPES, pH 7.4 (buffer A). Cells were scraped on ice, collected in buffer A containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, and 10 mM EDTA), and pelleted by centrifugation at 1300 x g for 10 min. Cells were resuspended in 0.6 ml of buffer A containing 1% Nonidet P-40, 20% glycerol, and the above protease inhibitors (buffer B), incubated on ice for 15 min, and diluted with 5.4 ml of buffer A containing 20% glycerol plus the protease inhibitors (buffer C). The mixture was centrifuged at 100,000 x g for 60 min. The supernatant (500 µl) was mixed with 100,000 cpm of <sup>125</sup>I-FSH and 6.5 µl of 0.9% NaCl and 10 mM Na<sub>2</sub>HPO<sub>4</sub> at pH 7.4 containing increasing concentrations of unlabeled FSH. After incubation for 12 h at 4 °C, the solution was thoroughly mixed with 250 µl of buffer A containing bovine γ-globulin (5 µg/ml) and 750 µl of buffer A containing 20% polyethylene glycol 8000. After incubation for 10 min at 4 °C, samples were pelleted at 1,300 x g for 30 min and supernatants removed. Pellets were resuspended in 1.5 ml of buffer A containing 20% polyethylene glycol 8000, centrifuged, and counted for radioactivity. Monoclonal anti-FSHR antibodies were radioiodinated and used for binding to nonbinding mutant FSHRs expressed on the intact cell surface as described previously (Hong et al., 1998).

## **Derivatization and Radioiodination of Peptides**

In the dark, 30 µg of receptor peptides in 40 µl of 0.1 M sodium phosphate, pH 7.5, was mixed with 1 mCi of Na-[<sup>125</sup>I]iodine in 10 µl of 0.1 M NaOH and 7 µl of chloramine-T (1 mg/ml) in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4. After 20 s, 7 µl of sodium metabisulfite (2.5 mg/ml) in 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, was introduced to terminate radioiodination. Radioiodinated peptides were mixed with 60 µl of 16% sucrose solution in PBS and fractionated on Sephadex Superfine G-10 column (0.6 x 15 cm) using PBS. Peptides were derivatized with 4-azidobenzoyl glycine (ABG) and radioiodinated as described previously (Sohn et al., 2002).

## **Photoaffinity Labeling of FSH**

The following solutions were sequentially introduced to siliconized glass tubes: 20 µl of 0.9% NaCl and 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, in PBS, 10 µl of FSH in PBS, and 10 µl of <sup>125</sup>I-FSHR<sup>9-40F13Bpa</sup> in PBS. Competitive inhibition experiments were carried out as described for the photoaffinity-labeling experiments with the exception that 10 µl instead of 20 µl of PBS was introduced to each tube and the mixture was incubated with 10 µl of increasing concentrations of nonradioactive receptor peptides. The mixtures were incubated at 37 °C for 90 min in the dark, irradiated with a Mineralight R-52 UV lamp for 3 min as described previously (Ji and Ji, 1980), and solubilized in 2% SDS, 100 mM dithiothreitol, and 8 M urea. The samples were electrophoresed on 8–12% polyacrylamide gradient gels. Gels were dried on filter paper and exposed to an imaging screen overnight, which was scanned on a PhosphorImager (Molecular Dynamics).

## **Deglycosylation**

The FSH  $\alpha$  and  $\beta$  subunits co-migrate on SDS-PAGE. To separate them on the gel, FSH was photoaffinity-labeled and deglycosylated with PNGase F. Enzymatic cleavage was done by incubation of the labeled FSH complex with 20 or 50 units of PNGase F (New England BioLabs) in 40  $\mu$ l for 18 h at 37 °C. The samples were solubilized in SDS under the reducing condition and electrophoresed on 15% gel containing 9 M urea.

## **Immunoblot of FSH Subunits**

Separated proteins were blotted onto 0.2- $\mu$ m nitrocellulose membrane as described previously (Towbin et al., 1979). Membranes were treated for 1 h with 5% blocking buffer (25 mM Tris-HCl, 1.4 M NaCl, 5% nonfat dry milk, 0.2% sodium azide, 1% Nonidet P-40, pH 7.4) and incubated with polyclonal anti-FSH and anti-FSH  $\alpha$  and  $\beta$  antibodies (dilution 1:2000 and 1:3500 each in blocking buffer) for 1 h at room temperature. Membranes were washed three times (5 min each) with the blocking buffer and incubated with anti-rabbit peroxidase-conjugated IgG (dilution 1:5000 in the blocking buffer) for 1 h at room temperature. Membranes were washed three times (5 min each) with the blocking buffer and twice (5 min each) with 25 mM Tris-HCl, pH 7.4. Membranes were incubated in staining solution (0.05% 3,3'-diaminobenzidine, 0.02%  $\text{CoCl}_2$ , 0.03%  $\text{H}_2\text{O}_2$ ) until bands became visible.

## RESULTS

### Activities of the N-terminal Region

In a first step to check the importance of the N-terminal region of human FSHR, each amino acid of the <sup>8</sup>SNRVFLCQESKVTEIPSDLPRNAIE<sup>33</sup> sequence was individually substituted with Ala. This sequence is diverse among the glycoprotein hormone receptors (Figure 3.2), although these receptors share a high overall homology and structural similarity (Ji et al., 1998). In contrast, the FSHR sequence is highly conserved among species, implicating its importance. HEK 293 cells were stably transfected with mutant receptor plasmids and selected for stably expressing individual mutant receptors. These cells were assayed for <sup>125</sup>I-FSH binding and FSH-dependent cAMP induction. The results show that some of the Ala substitutions significantly impacted the hormone binding and/or cAMP induction.

Ala substitutions for Ser<sup>9</sup>, Asn<sup>10</sup>, Arg<sup>11</sup>, Val<sup>12</sup>, Phe<sup>13</sup>, and Leu<sup>14</sup> improved FSH binding (Figure 3.3, A and B), FSH-dependent cAMP induction (Figure 3.3.C), or both. The K<sub>d</sub> values of FSHR<sup>N10A</sup>, FSHR<sup>R11A</sup>, and FSHR<sup>L14A</sup> were lower than the wild type value as were the EC<sub>50</sub> values of FSHR<sup>S9A</sup>, FSHR<sup>N10A</sup>, FSHR<sup>V12A</sup>, FSHR<sup>F13A</sup>, and FSHR<sup>L14A</sup> (see tables in Figure 3.3). Ala substitutions for Gln<sup>16</sup>, Glu<sup>17</sup>, Lys<sup>19</sup>, and Val<sup>20</sup> did not impact the EC<sub>50</sub> values and maximal cAMP induction (Figure 3.3.F), and K<sub>d</sub> values of the mutants were similar to or somewhat higher than the wild type value (Figure 3.3, D and E). In contrast, the S18A substitution resulted in a considerably lower EC<sub>50</sub> value despite a higher K<sub>d</sub> value. These results show an improved cAMP induction despite a lower hormone binding affinity and suggest an interesting and potentially crucial role of Ser<sup>18</sup> in modulating signal generation.

Ala substitution for Thr<sup>21</sup>, Glu<sup>22</sup>, or Ser<sup>25</sup> did not significantly impact hormone binding or cAMP induction (Figure 3.4). On the other hand, the I23A substitution partially impaired the cAMP induction with a 23-fold higher EC<sub>50</sub> value and 2.6-fold lower maximal

cAMP level. The P24A, D26A, and L27A substitutions completely abrogated hormone binding and therefore cAMP induction, suggesting the importance of these residues and this region. The P28A, R29A, N30A, A31G, I32A, and E33A substitutions did not dramatically impact the  $K_d$  and  $EC_{50}$  values or the maximal cAMP production (Figure 3.4). The nonbinding mutants were either incapable of binding the hormone or trapped in cells, and these possibilities have successfully been tested by assaying hormone binding after solubilization of cells in nonionic detergents (Hong et al., 1998; Hong et al., 1997; Rozell et al., 1995). The binding assay for receptors solubilized in Nonidet P-40 showed that FSH did not bind to any of the C15A, P24A, D26A, and L27A mutants (Figure 3.5.A), indicating that they are incapable of hormone binding. However, the result does not necessarily prove the surface expression of the nonbinding mutants and nonbinding on the surface. Therefore, the cells expressing them were probed with  $^{125}\text{I}$ -anti-FSHR antibody. The antibody bound to the cells (Figure 3.5.B), indicating the surface expression of the mutants and their inability to bind the hormone on the cell surface. These results, taken together, show several distinct effects of Ala substitutions as shown in the summary bar graph (Figure 3.6). C15A, P24A, D26A, and L27A abolished hormone binding. In contrast to these nonbinding mutations, N10A, R11A, and L14A improved hormone binding. On the other hand, I23A impaired cAMP induction by dramatically increasing the  $EC_{50}$  value. Remarkably, S9A, V12A, F13A, S18A, and I32A reduced the  $EC_{50}$  value by 2–3-fold while maintaining or slightly enhancing the maximum cAMP induction level. These results suggested the importance of this region of the receptor in hormone binding and cAMP induction and raised a question as to whether this region directly interacts with the hormone or indirectly impacts the global structure of the receptor.



## Photoaffinity Labeling of FSH

To examine the two general possibilities, a peptide mimic corresponding to the receptor sequence of  $^8\text{SNRVFLCQESKVTEIPSDLPRNAIELRFVLTK}^{40}$  was synthesized, FSHR<sup>9-40</sup> (Figure 3.7.A). A Tyr residue was attached to the N terminus for radioiodination, and the N terminus was acetylated while the C terminus amidated. Phe<sup>13</sup> was substituted with Bpa for photoaffinity labeling (Kauer et al., 1986). The ketone moiety of the Bpa group can be activated with UV at >350 nm and is capable of reacting with unreactive  $\alpha$ -CH bonds of amino acids (Bayley, 1983; Dorman and Prestwich, 1994; Kauer et al., 1986). To determine whether the resulting peptide  $^{125}\text{I-FSHR}^{9-40\text{F13Bpa}}$  could bind and photoaffinity-label FSH, it was incubated with FSH and irradiated with UV for increasing time periods. Samples were solubilized in SDS under reducing conditions and then electrophoresed. The autoradiographic phosphorimage of the gel (Figure 3.7.B) revealed labeling of the FSH band. The autoradiograph suggests that the two subunits of human FSH comigrated. The band was not labeled when the sample was not irradiated with UV, indicating the requirement for UV irradiation. The extent of the labeling was dependent on the irradiation time, reaching maximum labeling after 30-s irradiation. The result shows that the labeling is saturable. The hormone was labeled next with increasing concentrations of  $^{125}\text{I-FSHR}^{9-40\text{F13Bpa}}$  while maintaining FSH at a constant concentration (Figure 3.7.C). Conversely, increasing concentrations of FSH were labeled with a constant concentration of  $^{125}\text{I-FSHR}^{9-40\text{F13Bpa}}$  (Figure 3.7.D). If the labeling was specific, the concentrations should reach a plateau under both conditions. Indeed, the labeling plateaued under both conditions, indicating saturable and specific labeling of a substantial portion (>50%) of FSH. Furthermore, the labeling should be inhibited by nonradioactive peptide and unmodified wild type peptide. Therefore, FSH was incubated with  $^{125}\text{I-FSHR}^{9-40\text{F13Bpa}}$  in the presence of increasing concentrations of wild type peptide and nonradioactive FSHR<sup>9-40F13Bpa</sup> (Figure 3.7.E). The peptides inhibited

the photoaffinity labeling in a dose-dependent manner and eventually blocked the labeling. Peptides corresponding to exoloops 1–3, FSHR<sup>exo1</sup>, FSHR<sup>exo2</sup>, and FSHR<sup>exo3</sup>, were also tested. FSHR<sup>exo2</sup> inhibited the labeling as FSHR<sup>9–40</sup> did. In contrast, FSHR<sup>exo1</sup> inhibited the labeling with a ~30-fold less potency. On the other hand, FSHR<sup>exo3</sup> failed to block the labeling.

### Labeling Specificity

Although the photoaffinity labeling was specific for FSH, our data do not show the biological specificity of the affinity labeling. To address this concern, a constant amount of denatured FSH was incubated with increasing concentrations of <sup>125</sup>I-FSHR<sup>9–40F13Bpa</sup> and treated with UV. Denatured FSH was not labeled at all despite high concentrations of the peptide (Figure 3.8.A). Denatured FSH was not labeled when increasing concentrations of denatured FSH were incubated with a constant amount of <sup>125</sup>I-FSHR<sup>9–40F13Bpa</sup> and treated with UV (Figure 3.8.B). When FSH was denatured by boiling in 8 M urea for 30 min, it did not bind to FSHR and induce cAMP production. To test whether the denatured FSH remained in solution, the mixture of radioactively labeled FSH and unlabeled FSH was denatured and varying volumes of the mixture were transferred to other tubes and the radioactivity was counted. The transfer was quantitative with 99–100% efficiency, indicating that denatured FSH was present in the photoaffinity-labeling tube. These results indicate the specificity of the affinity labeling for biologically active FSH. To determine the labeling specificity, luteinizing hormone (LH), thyroid-stimulating hormone (TSH), growth hormone, phospholipase A, and urokinase were subjected to photoaffinity labeling with <sup>125</sup>I-FSHR<sup>9–40F13Bpa</sup> (Figure 3.8.C). None of them was labeled. If <sup>125</sup>I-FSHR<sup>9–40Bpa13</sup> specifically binds to FSH and labels it as indicated by the results, the peptide is expected to inhibit the in vivo binding of FSH to the receptor on intact cells. Indeed, FSHR<sup>9–40Bpa13</sup> and FSHR<sup>9–40</sup> inhibited <sup>125</sup>I-FSH binding to the receptor (Figure 3.8.D).

### Labeling of FSH $\beta$ Subunit

Because the two subunits of purified human FSH appeared to comigrate on SDS-PAGE, it was unclear which of the subunits was labeled. To resolve the subunits, FSH was deglycosylated with PNGase F and electrophoresed (Figure 3.9.A, lane 2). The two subunits were clearly separated into two distinct bands. Because the  $\beta$  subunit is larger than the  $\alpha$  subunit, the upper band is probably the  $\beta$  subunit. To conclusively determine the identity of the upper band, deglycosylated FSH was electrophoresed, the gel was blotted on nitrocellulose membrane, and the membrane was probed with anti-FSH  $\alpha$  and anti-FSH  $\beta$  antibodies. Anti-FSH  $\alpha$  antibody conspicuously labeled the lower band, whereas the anti-FSH  $\beta$  antibody recognized primarily the upper band and faintly the lower band (Figure 3.9, A and B). These results show that the lower band represents the FSH  $\alpha$  subunit, whereas the upper band is the FSH  $\beta$  subunit, indicating that FSH  $\beta$  was labeled. To compare this labeling of FSH  $\beta$  with the N-terminal peptide, the exoloop 3 peptide, FSHR<sup>ex03</sup>, was used for labeling FSH. The peptide was derivatized with a UV-activable reagent, ABG, and radioiodinated. The resulting <sup>125</sup>I-ABG-FSHR<sup>ex03</sup> was incubated with FSH, irradiated with UV, deglycosylated, and electrophoresed. The labeled FSH subunit appeared in the lower band, indicating the labeling of the FSH  $\alpha$  subunit (Figure 3.9.C) as reported previously (Sohn et al., 2002).

The labeling of FSH  $\beta$  by <sup>125</sup>I-FSHR<sup>9-40F13Bpa</sup> contrasts the labeling of the FSH  $\alpha$  subunit by the FSHR exoloop 3 peptide. If this contrasting labeling is specific and reflects the true interaction between FSH and the receptor, some of the peptides representing parts of the  $\alpha$  subunit sequence might inhibit the labeling. Particularly, if some of the  $\alpha$  peptides block the labeling of the  $\alpha$  subunit but not the  $\beta$  subunit, the result would support the selective

labeling results and show labeling specificity. Four  $\alpha$ -peptides,  $\alpha^{1-15}$ ,  $\alpha^{26-46}$ ,  $\alpha^{61-75}$ , and  $\alpha^{81-92}$  were tested, and none of them inhibited the labeling of the FSH  $\beta$  subunit by  $^{125}\text{I}$ -FSHR<sup>9-40F13Bpa</sup> (Figure 3.10.A). In contrast, the labeling of the FSH  $\alpha$  subunit by  $^{125}\text{I}$ -ABG-FSHR<sup>ex03</sup> was blocked by  $\alpha^{26-46}$  and somewhat by  $\alpha^{61-75}$  (Figure 3.10.B).  $\alpha^{1-15}$  and  $\alpha^{81-92}$  Peptides failed to block the labeling. These results support the differential labeling and its selectivity and validity.

## DISCUSSION

The Ala-scanning results indicate that the Ser<sup>9</sup>-Glu<sup>33</sup> sequence of the FSH receptor is important for surface expression, hormone binding, and signal generation. The photoaffinity-labeling results show that FSHR<sup>9-40F13Bpa</sup> photoaffinity labels FSH but not LH, TSH, growth hormone, phospholipase A, and urokinase. The labeling is saturable and dependent on the concentrations of FSH and derivatized  $^{125}\text{I}$ -FSHR<sup>9-40</sup>, UV activation, and UV exposure time.  $^{125}\text{I}$ -FSHR<sup>9-40F13Bpa</sup> photoaffinity labels bioactive FSH but not denatured hormone, and the labeling is blocked by nonderivatized wild type peptide and nonradioactive FSHR<sup>9-40F13Bpa</sup>. The labeling specificity is further underscored by the fact that it labeled the  $\beta$  subunit but not the  $\alpha$  subunit. These results suggest that the N-terminal region of the FSH receptor is in close proximity to FSH, probably interacting with the hormone. This conclusion is consistent with the previous reports that some residues of the region are important for hormone binding and receptor trafficking (Bradbury et al., 1997; Nechamen and Dias, 2000; Nechamen and Dias, 2003) and that the similar region of the LH receptor interacts with human chorionic gonadotropin (Hong et al., 1998; Phang et al., 1998).

In contrast to the labeling of the  $\beta$  subunit by the N-terminal peptide, the exoloop 3 peptide labeled the  $\alpha$  subunit. The significance of these contrasting results is 2-fold. First, it supports the validity and specificity of the photoaffinity labeling. Second, it provides the crucial information on the overall arrangement of the ternary complex involving the exodomain, FSH, and endodomain. The results suggest that the  $\beta$  subunit is near the N-terminal region of the exodomain, whereas the  $\alpha$  subunit is close to the exoloops of the endodomain. These are consistent with the previous reports that only the  $\alpha\beta$  dimer is capable of high affinity binding to receptors and inducing biological responses (Pierce and Parson, 1981; Roche and Ryan, 1985). Interestingly, all glycoprotein hormones utilize similar if not identical signal pathways consisting of adenylyl cyclase and phospholipase C $\beta$ . Therefore, the  $\alpha$  subunit has been implicated in the signal generation (Pierce and Parson, 1981).

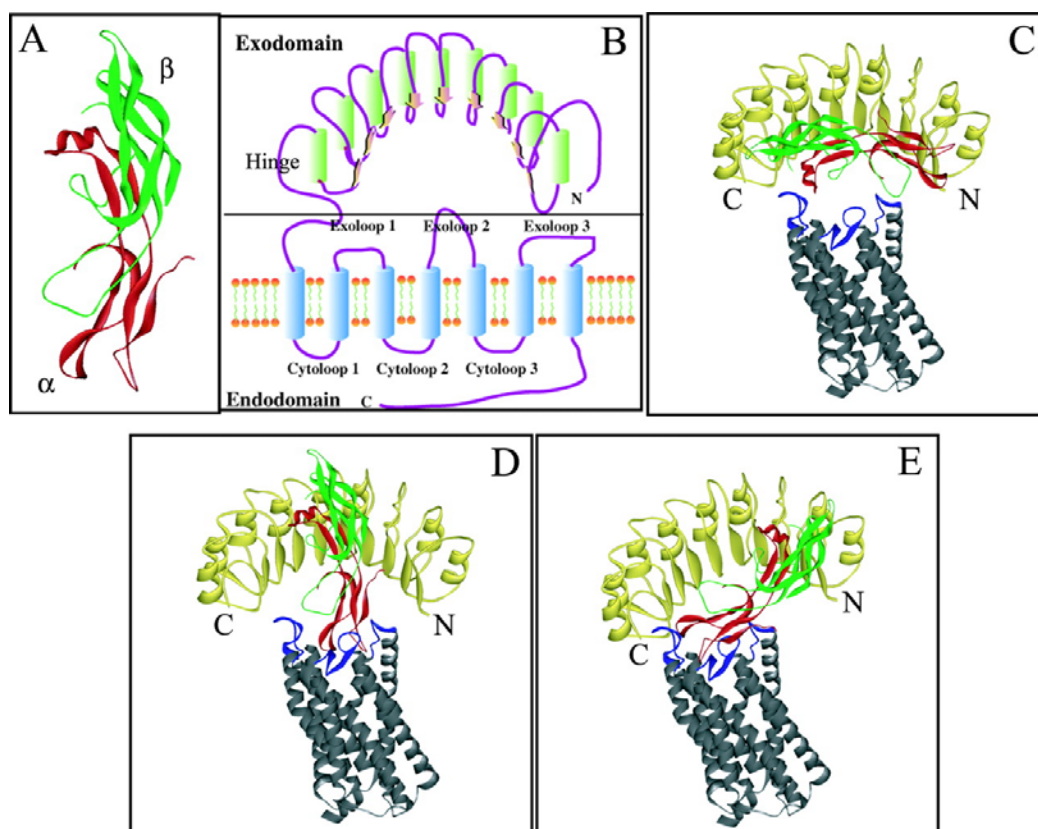
Based on these results, it is now possible to project the hormone interacting with both the exodomain and endodomain. Furthermore, they suggest that the hormone is probably in a vertically tilted position with respect to LRRs of the exodomain and the endodomain. To help visualize the arrangement and facilitate modeling of the ternary complex, one of several possible models is presented in Figure 3.1.E. The model suggests that parts of the  $\alpha$  subunit might interact with LRRs and, conversely, that some parts of the  $\beta$  subunit may be close to the exoloops. Such interactions could be probed by strategically attaching a photoactivable group at appropriate positions of the hormone subunits and/or using a reagent that can reach farther than ABG and Bpa. These two reagents can reach 10 and 7 Å, respectively. Other additional information will also be necessary to more precisely define the ternary structure. For example, the crystal structure of FSH shown in the model does not likely represent its structure in the ternary complex, because the gonadotropin undergoes conformational changes, particularly the interaction between the two subunits upon the initial interaction with

the receptor (Ji et al., 1995a). These conclusions are consistent with observations that the original quaternary structure of unbound hormone dimers is not essential for hormone action (Garcia-Campayo et al., 2002; Jackson et al., 1999).

The results that Ala substitution for some N-terminal residues improved hormone binding, cAMP induction or both suggest an interesting possibility that this region is involved in modulating not only hormone binding but also signal generation. The most dramatic improvement is seen in the S18A substitution, which improved the EC<sub>50</sub> value of cAMP induction by 3-fold as compared with the wild type value. Additionally, the maximum level of cAMP production only slightly increased. These observations indicate that the affinity and maximum level of cAMP induction are distinctly regulated. They suggest that FSH activates FSHR<sup>S18A</sup> more effectively than the wild type receptor does, which in turn results in better activation of the G protein. It will be interesting to see whether the number of activated G protein molecules is the limiting factor. The improved EC<sub>50</sub> is not related to the hormone binding affinity because the binding affinity of the mutant is somewhat less than the wild type affinity. These novel observations suggest an intriguing possibility that FSHR<sup>S18A</sup> is more sensitive to hormone binding and is capable of activating the G protein with higher affinity without significantly impacting the level of activation. Because the exodomain is likely to modulate the endodomain to generate hormone signals at the exoloops (Dufau, 1998; Ji et al., 1998; Ji et al., 1995b; Nishi et al., 2002; Zeng et al., 2001), a simple possibility is that the affinity of the modulation at the interface between the exodomain and exoloops is improved in FSHR<sup>S18A</sup>. Several other Ala substitutions, S9A, V12A, and F13A, also showed similar yet less dramatic results.

In conclusion, the evidence is presented that in the ternary exodomain/FSH/endodomain complex, FSH is vertically oriented with the  $\beta$  subunit close to

the N-terminal region and the  $\alpha$  tip projecting toward the exoloop 3. This is consistent with the recent report of the crystal structure of FSH/FSHR complex (Fan and Hendrickson, 2005).



**Figure 3.1 Structure of FSH and receptor.**

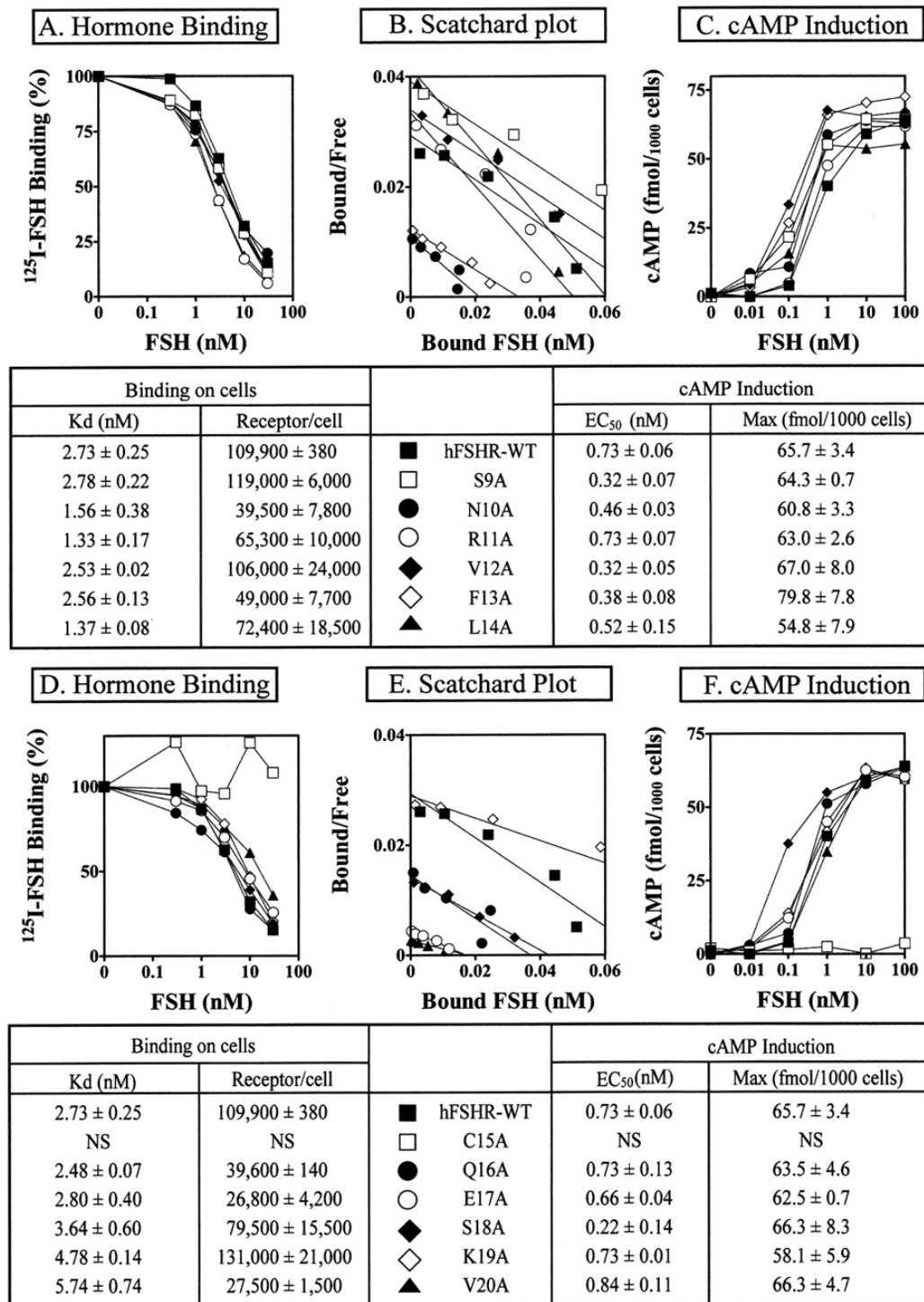
**A**, crystal structure of FSH, the  $\alpha$  subunit in red and  $\beta$  subunit in green. **B**, a schematic drawing of FSHR with two distinct domains, exodomain and endodomain. **C**, a schematic drawing of FSH interacting horizontally with LRRs of the exodomain. Note that the orientation of the  $\alpha$  and  $\beta$  subunits could be switched if the hormone is horizontally flipped. **D**, a schematic drawing of FSH interacting vertically with LRRs of the exodomain. Note that the orientation of the  $\alpha$  and  $\beta$  subunits could be switched if the hormone is vertically flipped. **E**, a hypothetical interaction of FSH  $\alpha$  and  $\beta$  with the LRRs of the exodomain and the exoloops of the endodomain. The FSH  $\beta$  subunit is close to the N-terminal region of the exodomain, whereas the FSH  $\alpha$  tip is projected toward the exoloops of the endodomain. The tilted orientation of FSH is based on this study.



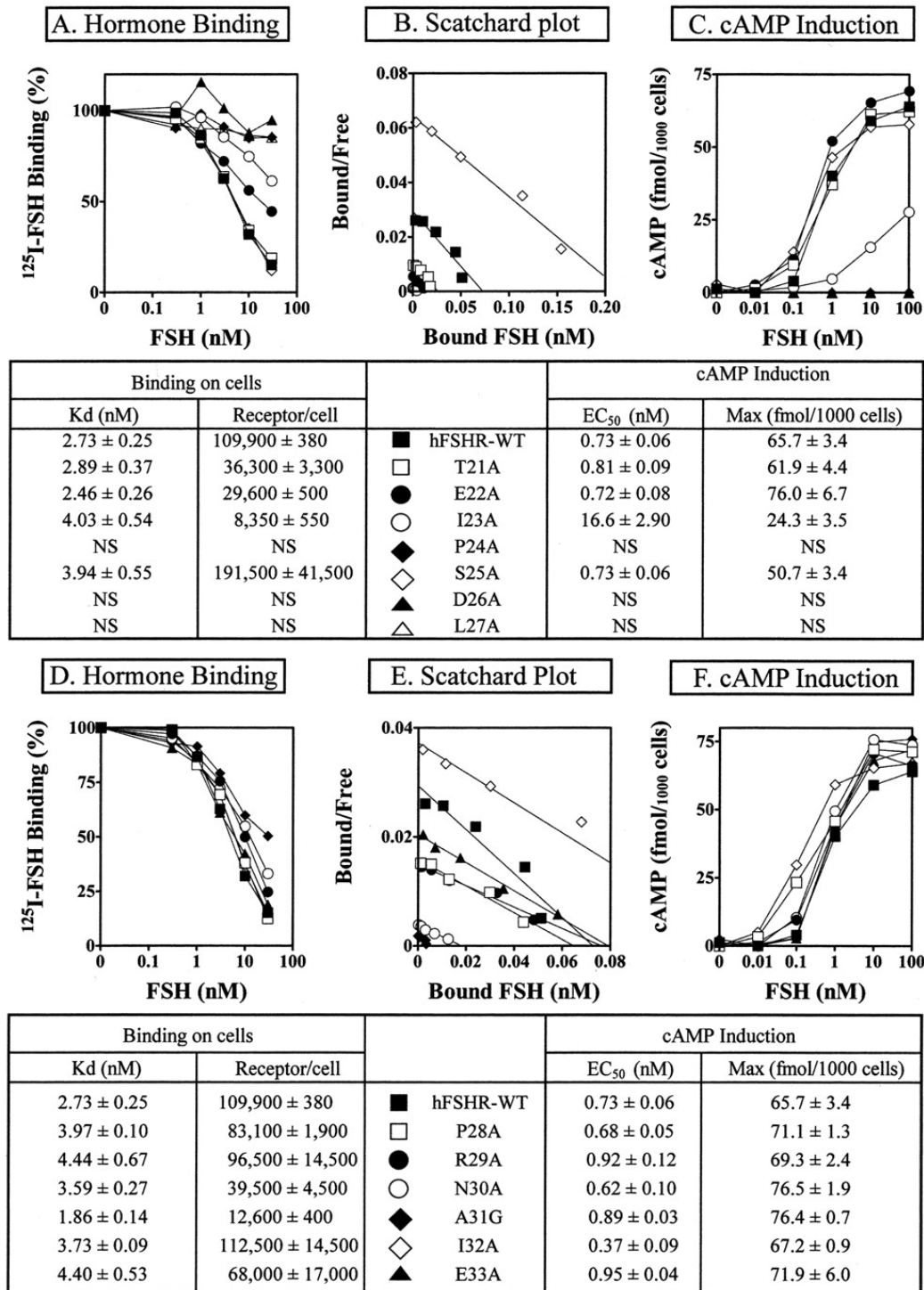
	10	15	20	25	30
Human	S N R V F L C Q E S K V T E I P S D L P R N A I E				
Rat	- - - - - - - D - - - - - - T - - - - - - -				
Bovine	- - G - - - - - - - - - - - - - - - D - V -				
Horse	- - - - - - - - - - - - - - - - - - L -				
Sheep	- - G - - - - - D - - - - - M - - - - - D - V -				
Pig	- - G - - - - - - - - - - - P - - - - - V -				
hLHR	P E P C N C V P D G A L R C P G P T A G L T R L S				
hTSHR	H Q E E D F R V T C - D I Q R I P S - - P S T Q T				

**Figure 3.2 Comparison of the primary sequence of the first 34 residues of the glycoprotein hormone receptors.**

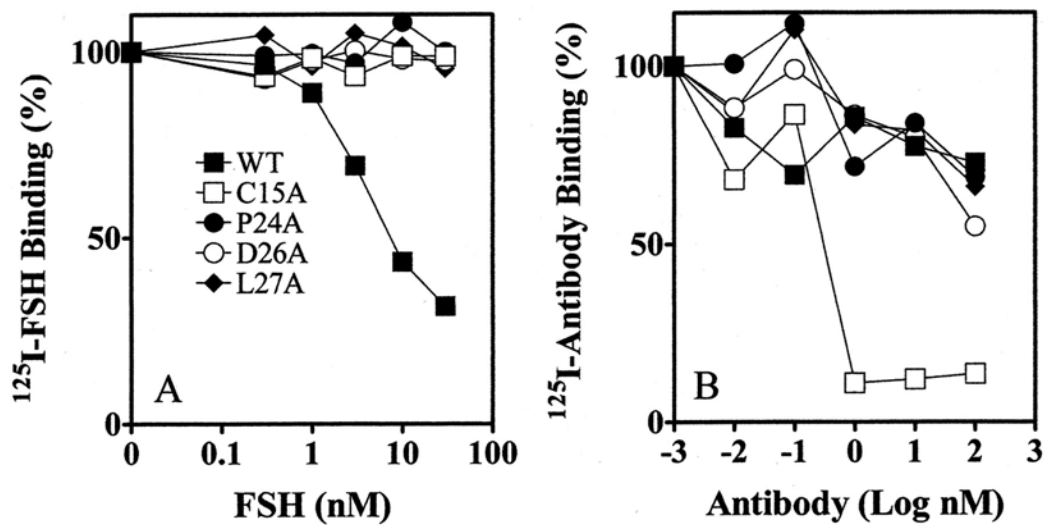
The FSH receptor sequences of various species were compared with the corresponding sequences of the human LH receptor and TSH receptor. Cys<sup>15</sup>, Ser<sup>18</sup>, Pro<sup>24</sup>, Asp<sup>26</sup>, and Leu<sup>27</sup> of FSHR are conserved among the species.



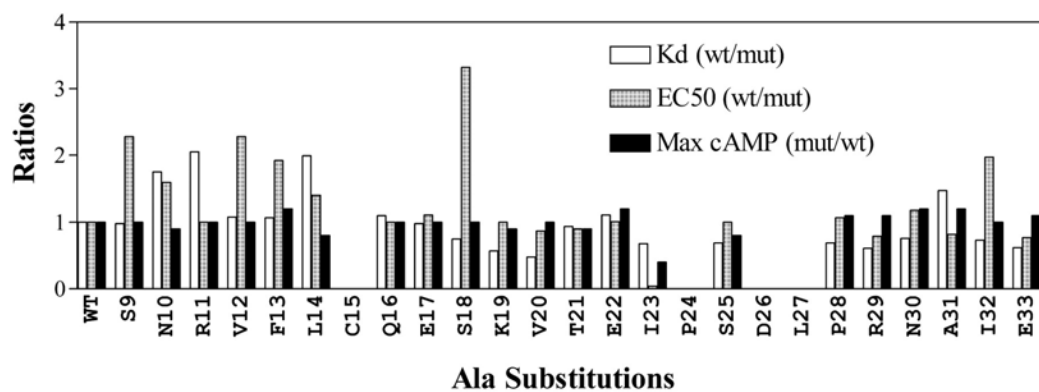
**Figure 3.3 Ala substitutions for Ser<sup>9</sup>-Val<sup>20</sup>.** A–F, residues from Ser<sup>9</sup> to Val<sup>20</sup> of the FSH receptor were individually substituted with Ala, and the resulting mutant receptors were stably expressed in HEK 293 cells. Intact cells were used for <sup>125</sup>I-FSH binding in the presence of increasing concentrations of unlabeled FSH (A and D) and for cAMP production (C and F). The competitive inhibition data (A and D) were converted to Scatchard plot (B and E). Experiments were repeated 4–6 times in duplicate, and the means ± S.D. were calculated. NS, not significant.



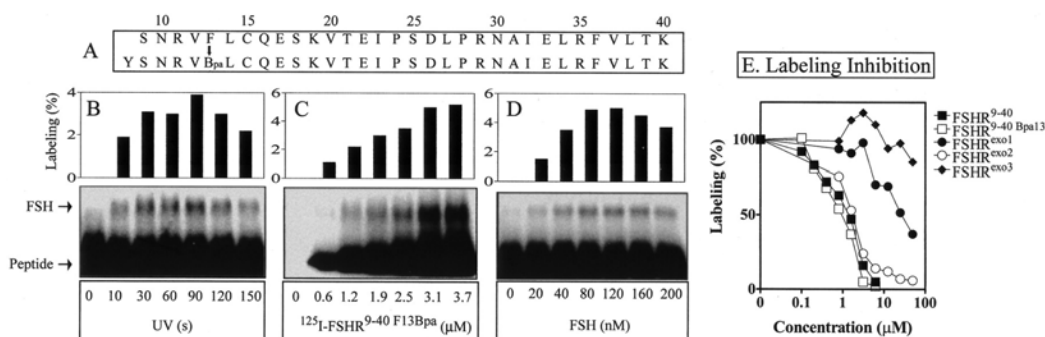
**Figure 3.4 Ala substitutions for Thr<sup>21</sup>-Glu<sup>33</sup>.** A–F, residues from Thr<sup>21</sup> to Glu<sup>33</sup> of the FSH receptor were individually substituted with Ala, and the resulting mutant receptors were expressed in HEK 293 cells and assayed as described in the legend to Figure 3.3.



**Figure 3.5 Hormone binding in solution and anti-FSHR antibody binding to intact cells.** A, cells individually transfected with the C15A, P24A, D26A, or L27A mutant receptor were solubilized in Nonidet P-40 and assayed for  $^{125}\text{I}$ -FSH binding as described under "Experimental Procedures." B, intact cells were also probed with  $^{125}\text{I}$ -labeled monoclonal anti-FSHR 106.105 antibody for the surface expression of the nonbinding mutants.

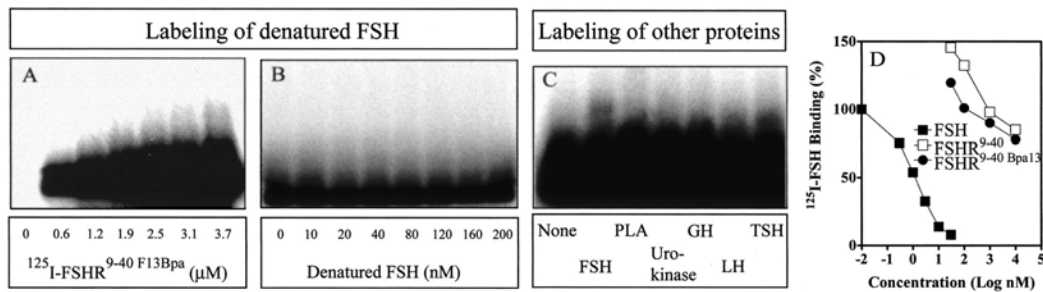


**Figure 3.6 Comparison of Ala substitution mutations.** To easily compare the activities of the wild type and mutant receptors, the ratios of  $K_d$  wild type mutant,  $EC_{50}$  wild type mutant, and maximum cAMP mutant wild type were presented in a bar graph.



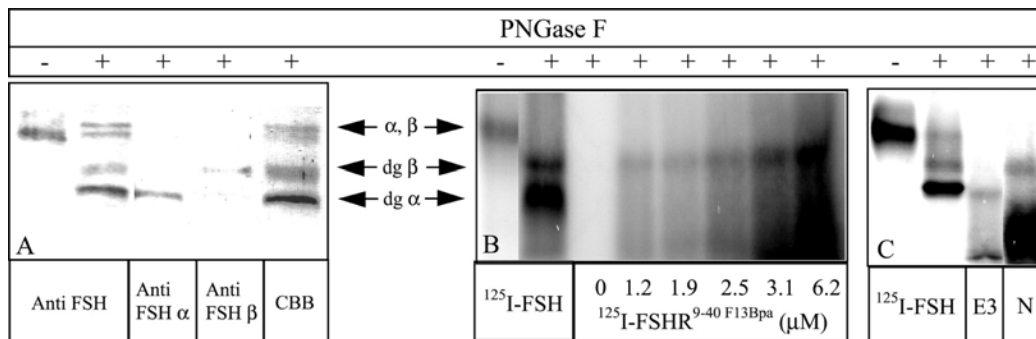
**Figure 3.7 Photoaffinity labeling of FSH with photoactivable FSHR<sup>9-40</sup>F13Bpa.**

The FSH receptor peptide corresponding to the sequence Ser<sup>9</sup>-Lys<sup>40</sup> (FSHR<sup>9-40</sup>) was synthesized with a Tyr at the N terminus for radioiodination and Bpa at the position of Phe<sup>13</sup> for photoaffinity labeling (A). The peptide was radioiodinated, and the resulting <sup>125</sup>I-FSHR<sup>9-40</sup>F13Bpa was incubated with FSH and irradiated with UV. B, the sample was irradiated with UV for increasing time periods from 0 to 150 s, solubilized in SDS under the reducing condition, and electrophoresed on polyacrylamide gel. After drying gels, they were exposed to a phosphorimaging screen and scanned on a PhosphorImager. The peptide appeared as the lower band, and the FSH α and FSH β subunits comigrated and appeared in the upper band. The intensity of each band in a gel lane was measured, and the percentage of the labeled FSH band in a gel lane was calculated based on the total intensity of a gel lane and presented in the bar graph above the autoradiograph. C, increasing amounts of <sup>125</sup>I-FSHR<sup>9-40</sup>F13Bpa from 0 to 3.7 μM were incubated with a constant amount (0.1 μM) of FSH and photolyzed for 60 s. The samples were processed as described above. D, increasing amounts of FSH from 0 to 0.2 μM were incubated with a constant amount (3.1 μM) of <sup>125</sup>I-FSHR<sup>9-40</sup>F13Bpa. E, FSH (80 nM) was incubated with 3 μM <sup>125</sup>I-FSHR<sup>9-40</sup>F13Bpa in the presence of increasing concentrations of unlabeled FSHR peptides, FSHR<sup>9-40</sup>, FSHR<sup>9-40</sup>F13Bpa, exoloop 1 peptide (FSHR<sup>exo1</sup>), exoloop 2 peptide (FSHR<sup>exo2</sup>), and exoloop 3 peptide (FSHR<sup>exo3</sup>). The samples were irradiated and processed as described in B.



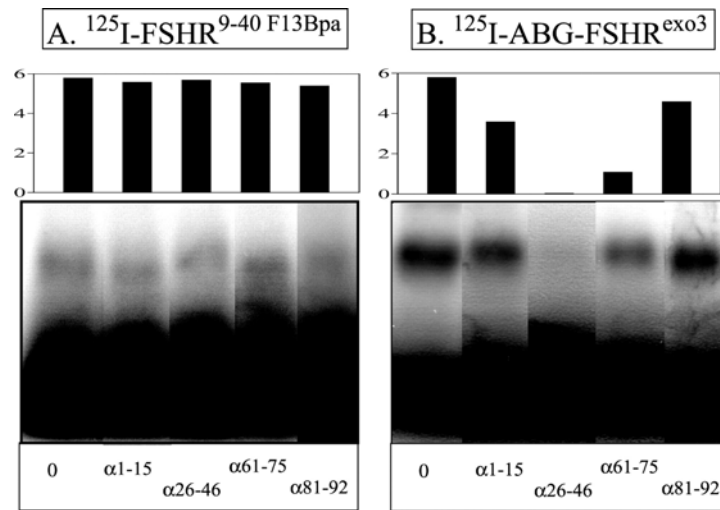
**Figure 3.8 Photoaffinity labeling of denatured FSH and other glycoproteins.**

**A**, increasing concentrations of  $^{125}\text{I}$ -FSHR<sup>9-40</sup>F13Bpa were incubated with 80 nM denatured FSH, irradiated with UV, and processed as described in the legend to Figure 3.7. FSH was denatured by boiling in 8 M urea for 30 min. **B**, a constant amount of  $^{125}\text{I}$ -FSHR<sup>9-40</sup>F13Bpa was incubated with increasing concentrations of denatured FSH, treated with UV, and processed. **C**, a constant amount of  $^{125}\text{I}$ -FSHR<sup>9-40</sup>F13Bpa was incubated with 5 nM each of FSH, phospholipase A (PLA), urokinase, growth hormone (GH), LH, or TSH, treated with UV, and processed as described in the legend to Figure 3.7. **D**, cells stably expressing FSHR were incubated with  $^{125}\text{I}$ -FSH and washed as described in Figure 3.3 but in the presence of increasing concentrations of FSHR<sup>9-40</sup> or FSHR<sup>9-40</sup>F13Bpa. Cells were washed three times and counted for the bound radioactivity.



**Figure 3.9 Immunoblot of FSH  $\alpha$  and  $\beta$  subunit bands.** **A**, FSH was treated with PNGase F, solubilized in SDS under the reducing condition, and electrophoresed along with nondeglycosylated FSH. Gel lanes were either stained with Coomassie Brilliant Blue (CBB) or blotted and stained with anti-FSH, anti-FSH  $\alpha$ , or anti-FSH  $\beta$  antibodies. **B**, FSH was incubated with increasing concentrations of  $^{125}\text{I-FSHR}^{9-40 \text{ F13Bpa}}$ , treated with UV, deglycosylated with PNGase F, solubilized, and electrophoresed along with  $^{125}\text{I-FSH}$ . **C**, the peptide corresponding to exoloop 3 ( $\text{FSHR}^{\text{ex}03}$ ) was derivatized with a UV-activable reagent, ABG, and radioiodinated. The resulting  $^{125}\text{I-ABG-FSHR}^{\text{ex}03}$  was incubated with FSH, treated with UV, deglycosylated with PNGase F, solubilized, and electrophoresed (E3). E3 was compared with FSH labeled with  $^{125}\text{I-FSHR}^{9-40 \text{ F13Bpa}}$  (N).





**Figure 3.10 Inhibition of photoaffinity labeling of FSH.** Peptides corresponding to the  $\alpha$  subunit sequences, 1–15, 26–46, 61–75 and 81–92, were synthesized. FSH was incubated with either  $^{125}\text{I}$ -FSHR<sup>9-40</sup>F13Bpa or  $^{125}\text{I}$ -ABG-FSHR<sup>exo3</sup> in the presence of an excess amount of  $\alpha$ <sup>1-15</sup>,  $\alpha$ <sup>26-46</sup>,  $\alpha$ <sup>61-75</sup>, or  $\alpha$ <sup>81-92</sup>. The samples were irradiated with UV and processed, and the percentage of the labeled FSH was calculated above the autoradiographs as described in Figure 3.7.

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## **PUBLICATIONS**

1. **Sohn J.**, Youn H.S., Jeoung M., Koo Y.B., Yi C.S., Ji I. and Ji T.H. **(2003)** Orientation of follicle-stimulating hormone (FSH) subunits complexed with the FSH receptor. Beta subunit toward the N terminus of exodomain and alpha subunit to exoloop 3. *J. Biol. Chem.* Nov 28;278(48):47868-76.
2. **Sohn J.**, Ryu K., Sievert G., Jeoung M., Ji I. and Ji T.H. **(2002)** Follicle stimulating hormone interacts with exoloop 3 of the receptor. *J. Biol. Chem.* Dec 20;277(51):50165-75.

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4. **Sohn, J.**, Mykles, D. L. and Cooper, R. L. (2000) Characterization of muscles associated with the articular membrane in the dorsal surface of the crayfish abdomen. *J. Exp. Zool.* Oct 1;287(5):353-77.

### **ABSTRACTS/TALKS**

1. **Sohn, J.**, Youn, H.S., Ji, I. and Ji, T.H. (2004) Orientation of FSH subunits complexed with the FSH Receptor. *UK repro. Forum Abstr. p.30* (Poster presented at the 23th Univ. of Kentucky Repro. Forum, May 20-21, 2004.)
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3. **Sohn, J.**, Youn, H.S., Ji, I. and Ji, T.H. (2002) The Amino-terminal region of the FSH receptor contacts FSH. *UK repro. Forum Abstr. p.34* (Poster presented at the 21th Univ. of Kentucky Repro. Forum, May 17-18, 2002.)
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5. **Sohn, J.** and Cooper, R.L. (1999) The anatomical and physiological characterization of muscles in the dorsal surface of the crayfish abdomen. *Am. Zool.* 38:202A.

### **AWARDS**

2002-2005                      Reproductive Science Training Program (RSTP) NIH Traineeship